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Tartrate-resistant acid phosphatase: prognosis in colorectal cancer and the M1/M2
distinction.

A thesis submitted to
the Yale University School of Medicine
in partial fulfillment of the requirements for the
Degree of Doctorate of Medicine

By
Chi-Joan How
Class of 2015

Abstract

Recent research has indicated that separate populations of macrophages are associated with differing outcomes in cancer survival. In our study, we examine macrophage expression of tartrate resistant acid phosphatase (TRAP) and its effect on survival in colon cancer. Immunohistochemical analysis on colorectal adenocarcinomas confirmed macrophage expression of TRAP. Co-localization of TRAP with CD68, a pan-macrophage marker, revealed that TRAP is present in some but not all subpopulations of macrophages. Further co-localization of TRAP with CD163, an M2 marker, revealed that TRAP is expressed by both M2 and non-M2 macrophages. TRAP expression was then measured using the AQUA method of quantitative immunofluorescence in a tissue microarray consisting of 233 colorectal cancer patients seen at Yale-New Haven Hospital. Survival analysis revealed that patients with high TRAP expression have a 22% increase in 5-year survival (uncorrected log rank $p=0.025$) and a 47% risk reduction for disease specific death ($p=0.02$). This finding was validated in a second cohort of older cases consisting of 505 colorectal cancer patients. Patients with high TRAP expression in the validation set had a 19% increase in 5-year survival (log rank $p=0.0041$) and a 52% risk reduction of death ($p=0.0019$). TRAP expression was also significantly associated with brisk rather than non-brisk tumor-infiltrating lymphocytes. These results provide evidence that macrophage expression of TRAP is associated with improved outcome, and implicates TRAP as a potential biomarker in colon cancer.

Acknowledgements

I would like to thank David L. Rimm for his mentorship and guidance throughout this project. I would also like to thank Jason R. Brown for his contributions and teaching in the lab. Thank you also to Sasha R. Saylor for her work in collecting all patient demographics, and Lori Charette and her team in YPTS for construction of the tissue microarrays used in this work. Special thanks to my friends, roommates, family, and fiancé, Sheng Si, for their never-ending love and support.

Contributions

TMA construction was performed at the Yale University TAM facility, with demographic and clinical information collected by S.S. D.L.R. and J.H. designed the experimental studies. Immunohistochemistry and immunofluorescence, including AQUA analyses and multiplexing of TRAP and CD68, CD163, and iNOS, was performed by J.H with guidance from J.R.B. Image acquisition was performed by J.H. Quantitative measurement of immunofluorescence was performed by J.H. using a stromal mask designed by J.R.B. All statistical analyses were performed by J.H.

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Introduction

Colorectal cancer (CRC) is the third-leading cause of cancer-related mortality in the United States, expected to cause 50,310 deaths in 2014.¹ Although many prognostic biomarkers have been reported, standard of care for colon cancer is still largely limited to assessment of TNM based staging, in which T denotes tumor depth of invasion, N denotes lymph node metastases, and M denotes distant metastasis. Staging is the most important prognostic factor in CRC, as Stage I CRC patients have estimated 5 year survival rates around 74%, Stage II between 37-67%, Stage III 28-73%, and Stage IV around 6%.¹ Given the wide range of survival outcomes even within a stage, the development of accurate prognostic markers has been an area of intensive research. Current clinically validated biomarkers found to be predictive and prognostic in CRC include candidate genes involved in the molecular carcinogenic pathways, including a KRAS mutation, B-RAF mutation, and mismatch repair deficiency.² There is also a wealth of tumor biomarkers not yet clinically validated, including but not limited to tumor-secreted pro-angiogenic factors (vascular endothelial growth factor 2 – VEGFR2) or receptor tyrosine kinases (epidermal growth factor receptor 2 – EGFR).²

Recent work has described the evaluation of the immune response as a prognostic indicator in CRC. The involvement of the immune system in carcinogenesis was first formally elucidated in 1957, when Thomas and Burnet theorized that lymphocytes served an important function in preventing the growth of newly transformed cancer cells.³ Further refinement of this theory of immunosurveillance has led to the modern-day concept of “immunoediting,” in which the host immune system detects and eliminates these cancer cells after intrinsic tumor suppressor mechanisms have failed.⁴ Support for

cancer immunoediting include studies that have shown a robust correlation between improved survival and the degree of tumor-infiltrating lymphocytes (TIL) in ovarian cancer, melanoma, and lung cancer.⁵⁻⁷ The impact of TIL on survival in CRC has also been robustly investigated, with multiple studies showing improved patient outcome in CRC with increased immune cell infiltration into tumor tissue.⁸

Further analysis of TIL has revealed more specific information on the type of immune cells associated with favorable prognosis in CRC. Using specific immunohistochemical markers in a large CRC patient cohort, Galon et al. found that a high density immune response consisting of memory CD4 cells and cytotoxic CD8 cells was associated with greater disease-free and overall survival.⁹ Furthermore, Naito et al. found that the specific location of CD8+ T cells relative to the tumor resulted in further increased survival, with intratumoral locations having more favorable prognoses than locations at the tumor margin.¹⁰ This suggests a more complex relationship between the presence of lymphocytes and tumor rejection. Indeed, the interaction between TILs with cancer cells is further modulated by aspects of the innate immune system, the tumor stroma, and the tumor cells themselves.^{10,11} In particular, macrophages have been found to be an important modulator of both the host immune response and tumor cell behavior.^{12,13}

Macrophages are monocyte-derived phagocytes originating from the bone marrow that subsequently migrate to diverse human tissues. Once present in their tissue microenvironment, they respond to a whole host of local signals and participate in a variety of specific functions, such as phagocytosis and clearance of debris, inflammation, pathogen defense, wound healing, and even tumor growth and/or suppression.¹⁴ In terms

of purported anti-tumor mechanisms, macrophages have been shown to be activated by the “inflammatory challenge” presented by cancer cells; upon encountering tumor cells, cytotoxic lymphocytes and natural killer cells release interferon-gamma, a potent activator of macrophages.¹⁵ In turn, macrophages exhibit unique anti-tumor mechanisms, including the release of enzymes, complement, cytokines, prostaglandins, and leukotrienes that are cytotoxic to tumor cells.¹⁶ The most well-studied of these secretions include tumor necrosis factor (TNF), which has experimentally been shown to damage transformed cancer cell lines, and interleukin-1 (IL-1), a cytokine essential for local inflammatory responses.¹⁷ In addition, both TNF and IL-1 exert feedback on activated lymphocytes and macrophages to augment the immune response against cancer cells.¹⁷ Studies have also shown cell-to-cell killing between macrophages and tumor cells; macrophages not only demonstrate phagocytic ability, but EM images have also captured non-phagocytic, lytic cell-to-cell killing of cancer cells by macrophages, with translocation of toxic macrophage lysosomal organelles into the cytoplasm of the tumor cell.^{18,19} Thus, while these cytotoxic functions of macrophages have been historically thought to target microbes and pathogens, they also are able to act against transformed cancer cells recognized by the immune system as aberrant.

Consistent with these mechanisms, there have been multiple studies that suggest improved survival outcome in CRC with increased macrophage infiltration.^{9,20-22} Using a pan-macrophage marker, CD68, Forssell et al. demonstrated that mean macrophage infiltration along the tumor front conferred a significant survival advantage independent of grade, stage, and lymphocytic infiltration.²⁰ Interestingly, Forssell et al. performed additional *in vitro* studies of co-cultured macrophage and colon cancer cell lines, in

which he demonstrated inhibited cancer cell growth with increased cell-to-cell contact of tumor cells with macrophages.²⁰ This is consistent with earlier findings of directly cell-mediated anti-tumor effects in macrophages. Similarly, Nagorsen et al. showed that both macrophage and dendritic cell infiltration in CRC tumors were associated with better prognoses, implicating the dendritic cell as an important mediator between the innate and adaptive immune systems due to its role in antigen presentation.²² This association between macrophage infiltration and improved survival is especially robust in CRC, but has been extended in prostate,²³ lung,²⁴ and stomach cancers.²⁵

However, the role of the macrophage in carcinogenesis is far more complex, as additional research has also implicated tumor-infiltrating macrophages in the role of tumor growth and development. These so-called “tumor-associated macrophages” (TAMs) are associated with significantly poorer clinical outcome in breast,^{26,27} lung,²⁸ bladder,²⁹ cervical cancer,³⁰ and Hodgkin’s lymphoma.³¹ This is due to the large repertoire of functions that macrophages have within its local microenvironment. Although macrophages may exhibit tumoricidal effects through the production of toxic intermediates such as nitric oxide (NO) or reactive oxygen species (ROS),³² macrophages are also able to exhibit tumorigenic effects through the production of growth factors,²⁶ promotion of angiogenesis,²⁷ and downregulation of inflammatory reactions.^{32,33}

For instance, although macrophages can be associated with microbial or tumor killing, its involvement in chronic inflammation also has been shown to promote tumor growth, spread, and invasion.³⁴ Macrophages secrete growth factors necessary for tumor development; in *in vitro* models, the close interactions between TAMs and carcinoma cells facilitate tumor invasion into blood vessels.^{26,35} For instance, TAMs and carcinoma

cells are capable of creating a paracrine loop, in which tumor cell production of colony-stimulating factor 1 (CSF-1) activate CSF-1 receptors on macrophages, and macrophage production of epidermal growth factor (EGFR) activate EGFR receptors on tumor cell.³⁵ This dual signaling in turn promotes movement of both the macrophage and tumor cell down CSF-1 and EGFR gradients toward blood vessels, and in breast cancer, has been associated with poor survival.²⁶ Paradoxically, while macrophage production of TNF in large amounts is associated with tumor cell destruction, the chronic release of TNF as seen in inflammation is associated with metalloprotease induction, allowing tissue remodeling and thus tumor growth through the surrounding stroma.³⁶ Macrophages have also been shown to secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF), nitric oxide synthase, and interleukin-8 (IL-8).³⁷ The hypoxic conditions of a tumor microenvironment may further stimulate macrophage production of pro-angiogenic factors. In *in vitro* models of subcutaneous melanoma, tumor growth rate and tissue capillary density were found to be increased in the presence of macrophages expressing angiotensin I receptors.³⁸

These divergent actions suggest that there exists different subpopulations of macrophages, each with its unique characteristics and functions, and each with its own role within the tumor microenvironment. Indeed, research has shown that the differentiation into these specific subsets of macrophages depends largely on both intrinsic and extrinsic cues in the local environment. Hematopoietic stem cells in the bone marrow give rise to myeloid precursors, which in turn mature into monocytes that enter into and circulate within the blood stream for 1-3 days.³⁹ During the “steady state,” these monocytes may differentiate into resident macrophages (alveolar macrophages, Kupffer

cells, etc.); however, under inflammatory conditions, these monocytes can also differentiate into inflammatory macrophages and dendritic cells.³⁹ It is unclear whether determination of the type of macrophage differentiation depends partially on intrinsic characteristics of the monocytes, or if macrophage differentiation occurs from a random pool of monocytes. Some studies have shown that certain cell receptors (in particular, cells of a CCR2 phenotype), are more prone to transformation into inflammatory versus residential macrophages, suggesting partial monocyte commitment.³⁹ However, local cytokine release from the inflammatory site exerts a large influence on the type of monocyte differentiation. Granulocyte macrophage colony stimulating factor (GM-CSF) for instance has been shown to affect differentiation into dendritic cells, whereas macrophage colony-stimulating factor (M-CSF) predisposes inflammatory macrophage formation.⁴⁰ These inflammatory macrophages can be further subspecialized into specific subpopulations, again based on the type of local signaling in the host tissue.

Further specialization of macrophages is largely due to the existence of two broad categories of inflammatory responses, each with its associated signature of cytokines. In type 1 immunity, T helper type 1 (Th1) lymphocytes stimulate phagocytic killing of microbes, a major component of cell mediated immunity.⁴¹ Type 2 immunity, mediated by T helper type 2 (Th2) lymphocytes, is characterized by suppression of phagocytic activity and activation of the humoral response, and historically is effective against parasites.⁴¹ Besides defense against infections caused by parasites, type 2 responses are necessary in the resolution of inflammation, and thus Th2 activated macrophages have enhanced “alternative” functions in tissue repair. This is in contrast to the phagocytic state of Th1 immune responses, in which macrophages are “classically” activated to

ingest foreign microbes. The presence of Th1 and Th2 responses has thus led to the broad classification of two types of macrophages. M1, or “classically activated” macrophages, are primarily involved in tissue destruction and microbial killing via the production of cytotoxic intermediates and activation of the Th1 type immune response.^{32,33} On the other hand, M2, or “alternatively activated” macrophages, consist of all non-classically activated macrophages, and are primarily involved in tissue remodeling, angiogenesis, and anti-inflammatory responses.^{32,33} The determination of M1 versus M2 differentiation is dependent on the local milieu of the host environment.^{32,33}

The differentiation of M1 and M2 macrophages has been an area of intensive study. As mentioned above, M1 macrophages are activated by Th1 lymphocytes and also natural killer cells. IFN-gamma is a particularly potent activator of M1 macrophages and is secreted by Th1 and NK cells.¹⁵ M1 macrophages are also activated by lipopolysaccharide (LPS) found on the surface of bacterial cell walls; other inducers of M1 differentiation include TNF-alpha and GM-CSF.⁴² In contrast, M2 macrophages are activated by Th2 lymphocytes; other activating signals include TFG-beta, glucocorticoids, M-CSF, IL4 and IL3.⁴³ Interestingly, IL4 and IL3 serve not only to activate M2 macrophages, but also inhibit the development of M1 macrophages.⁴³ Once differentiated, M1 and M2 macrophages exhibit unique expression signatures. M1 macrophages show high expression of ROS and intermediate nitrogen species, as well as inflammatory cytokines such as TNF alpha, IL-12, and IL-6.^{42,43} Due to the high levels of arginine synthesis, inducible nitric oxide synthase (iNOS) has been used as a biomarker for M1 macrophages; other common markers include CD40, CD64, and CCR7.⁴⁴ M2 macrophages express fewer pro-inflammatory cytokines, and function primarily in tissue

remodeling, angiogenesis, and limitation of the M1 immune response.^{42,43} These macrophages characteristically express IL-10, M2 chemokines, and scavenger, mannose, and galactose receptors, with common markers including CD163, CD204, and CD206.⁴⁴

The M1/M2 balance has important implications not just in pathogen response, but also in the host response to tumor cells. It has been hypothesized that while M1 macrophages promote tumor killing, M2 macrophages promote tumor growth, and the balance of M1 versus M2 macrophages has significant implications on cancer invasion and spread.^{32,45} A proposed “macrophage balance” cites that TAMs exhibit both inhibiting and activating effects on tumor growth, and the outcome of this interaction depends on both the types of macrophages recruited and the properties of the tumor cells.⁴⁶ The local tumor microenvironment is critical in determining the differentiation of recruited macrophages. For instance, secretion of IL-6 and M-CSF by renal cell carcinoma cell lines has been shown to preferentially transform recruited macrophages into phagocytic cells that lack antigen-presenting capability, effectively blocking any potential cytotoxic function.⁴⁷ Thus, for the majority of cancers, the local tumor microenvironment may predispose to an M2 phenotype, accounting for the estimation in one review that 80% of studies evaluating macrophage correlation with survival show worse outcome with increased TAMs, and 20% of studies show improved outcome with increased TAMs.⁴⁸ Interestingly, CRC makes up a large proportion of improved survival with increased macrophage infiltration, and some studies have suggested that CRC may recruit a larger proportion of M1 macrophages.⁴⁶ For instance, Bogels et al. found that monocytes cultured in supernatants of CRC cells exhibited a “secretome” that consisted of pro-inflammatory cytokines and ROS, whereas monocytes cultured in supernatants of

breast cancer cells showed increased expression of cytokines characteristic of an alternatively-activated phenotype.⁴⁶

Indeed, this “macrophage balance” has been bolstered by further studies that have shown that distinct subsets of macrophages are present in different tumor microenvironments,⁴⁹⁻⁵¹ and the distribution of M1 versus M2 activity is associated with variable outcomes and clinicopathological features.⁵²⁻⁵⁵ In a study by Heys et al. in breast cancer, macrophage SOCS3 expression was used as a proxy for M1 activation, and was found to be significantly associated with complete pathological responses to chemotherapy;⁵² similarly, Ohri et al. showed a significant survival advantage in non-small cell lung cancer patients with M1 macrophage infiltration of tumor islets.⁵⁴ Conversely, Medrek et al. showed that CD163+ (M2) macrophages in tumor stroma was significantly correlated with higher grade and larger tumor size in breast cancer,⁵³ and Kurahara et al. demonstrated poorer survival in pancreatic cancer patients with CD163+ TAMs at the invasive front.⁵⁵ In CRC, the M1/M2 distinction has been less clear in guiding prognosis, as while studies have shown improved survival with increased M1 macrophage infiltration, there is also improved survival with increased M2 macrophage infiltration as well.^{22,56} Indeed, more recent research has suggested that the M1/M2 distinction may be an oversimplification of macrophage phenotypes; macrophage differentiation may occur along a spectrum of M1 versus M2 activity, and macrophage differentiation itself may be plastic such that any given cell has the potential for multiple functions.³² The lumping of all non-classically activated macrophages as “M2 macrophages” also ignores the diverse functions within this class, which may account for the unclear association seen between M2 macrophage infiltration and CRC survival.⁵⁷

The implications of the M1/M2 distinction however go beyond prognostication, as there has been considerable interest in targeting macrophages and the tumor microenvironment in cancer immunotherapy. Given the evidence that TAMs are associated with poor outcomes in a large host of cancers, new strategies to block M2 activation or even reprogram TAMs into M1 phenotypes have emerged.^{58,59} For instance, the application of a CSF-1 antibody has led to decreased TAM presence in breast adenocarcinoma and slowed primary tumor growth in conjunction with paclitaxel treatment in mice.⁶⁰ Similarly, strategies to reprogram M2 macrophages into M1 phenotypes have included administration of antagonistic antibodies to IL-4 (a potent activator of the M2 phenotype),⁶¹ and agonist antibodies to CD40 (a stimulator of M1-type activity).⁶² Of particular interest is the programmed death 1 (PD-1) protein and its ligand, programmed death ligand 1 (PD-L1), which is abundantly expressed by macrophages and dendritic cells as well as on many tumors. PD-L1 is a major inhibitory ligand that induces suppresses T cell activation, and blockade of its interaction with PD-1 has led to improved immune responses and decreased tumor growth in vitro.⁶³ These observations have led to the development of anti-PD-L1 antibodies, with a Phase 1 clinical trial showing promising therapeutic results in patients with advanced cancer.⁶⁴ Thus, characterizing the different macrophage phenotypes in the tumor microenvironment has important implications in cancer therapeutics, as for instance PD-L1 expression on TAMs remains an attractive target for future cancer immunotherapy.

In this study, we investigate macrophage expression of tartrate-resistant acid phosphatase (TRAP) as a potential biomarker in colon cancer outcome. TRAP is a metalloprotease that catalyzes hydrolysis of phosphate esters.⁶⁵ It is highly expressed in

osteoclasts and was first discovered for its importance in bone resorption.⁶⁶ Clinically, TRAP has been used as a biomarker for growth and bone turnover and hairy cell leukemia.^{66,67} However, in addition to its roles in skeletal development, TRAP is highly expressed in activated macrophages and plays an important function in innate immunity. TRAP has been implicated in catalyzing the generation of ROS,⁶⁵ and it has been observed that macrophages overexpressing TRAP display increased superoxide production and bacterial killing.⁶⁸ In addition, certain substrates of TRAP have been shown to mediate Th1 type immunity, resulting in macrophage production of cytokines typical of M1 activity.^{69,70} Interestingly, recent research has suggested that tumor expression of TRAP is a negative prognostic marker in cancers with bone metastasis⁷¹ and melanoma.⁷² The mechanism of action, however is unknown. To date, we know of no examples of examination of TRAP expression as a prognostic indicator in the context of immune mediated responses to tumor invasion.

Purpose of Study

The purpose of this is to evaluate TRAP as a potential biomarker in CRC and determine its prognostic significance. Given TRAP's important role in innate immunity, we hypothesize that TRAP will be associated with improved outcome in CRC. We furthermore predict that TRAP will be associated with a specific subset of macrophages.

Materials and Methods

Tissue Microarrays and Patient Cohorts

Tissue microarrays (TMAs) for two separate and independent cohorts were constructed at the Yale University TMA facility (New Haven, CT) with formalin-fixed, paraffin-embedded tumor samples, as described previously.^{73,74} Briefly, multiple core needle biopsies from a variety of tumor tissues are embedded in a single block of paraffin, which can then be sectioned into tissue microarrays containing a high volume of individual patient samples.^{73,74} The more recent set contained 276 primary colorectal carcinomas from patients who were treated at Yale New Haven Hospital in New Haven, CT from 2000-2005. The earlier set contained 629 primary colorectal carcinomas from patients who were treated at Yale New Haven Hospital in New Haven, CT from 1970-1981. All follow-up information on the patients was obtained from the Yale New Haven Tumor Registry, the Yale-New Haven Hospital medical records and the Connecticut Death Records. Demographic and clinical information on each cohort is summarized in Table 1. Clinical and pathological information were taken at the time of TMA construction. For the purposes of this study, the newer cohort (YTMA 221) was used as the training set and the older cohort (YTMA 8) was used as the validation set. Disease-specific death was used to measure survival in the validation set, but due to lack of information on cause of death in the training set, alive-dead status was used to measure survival in the training set.

In order to assess whether there were any significant differences between the training and validation sets, we performed two-sample proportions and t-tests on each of the demographic and clinical characteristics. There were no significant differences in the

proportion of females ($p=0.28$) and the proportion of patients with Stage I ($p=0.56$), Stage II ($p=0.82$), Stage III ($p=0.62$) and Stage IV ($p=0.08$) colorectal carcinomas in each cohort. A significant difference was found in the mean patient age of each cohort ($p=0.03$); however, the mean patient age between the two cohorts differed by only about two years (training: 69.6 years; validation: 67.5 years). In addition, there were significant differences in the proportion of patients with well, moderately, and poorly differentiated colorectal carcinomas between the two cohorts ($p<0.01$). However, a survival analysis of the two cohorts revealed no significant differences in overall survival ($p=0.99$). These results indicate that the two cohorts were reasonably similar in major demographic and clinical characteristics. Table 1 and Figure 1 summarizes the above findings. Kaplan-Meier curves by stage for both the training and validation cohorts were performed, with significantly worse outcomes associated with increased stage for both cohorts ($p<0.001$).

TMAAs were constructed in two-fold redundancy for each cohort. Average redundancy in analysis was approximately 60%. A control (index) TMA containing 34 primary colorectal carcinomas from patients treated at Yale New Haven Hospital from 1970-1981 was used for run to run standardization.

Immunohistochemistry and Immunofluorescence

Immunohistochemical visualization of TRAP or CD68 was performed using a diaminobenzidine (DAB) staining protocol on serial index arrays. Slides were first deparaffinized by baking at 60°C for 30 minutes followed by 2x xylene treatment for 20 min each. Antigen retrieval was performed by pressure cooking with citrate buffer pH 6 at 97°C for 20 min. Slides were then permeabilized in 0.3% H₂O₂ in methanol for 30 min

Table 1. Characteristics of Colorectal Cancer Cohorts		
	New Set (2000-2005)	Old Set (1970-1981)
	YTMA-221	YTMA-8
Total Number	233	505
25% Survival, mo	30 (alive-dead)	25 (disease-specific death)
Age		
Range	30-97	23-94
Mean*	69.59	67.49
Median	≤72: 121 (51.9) >72: 112 (48.1)	≤68: 256 (50.7) >68: 249 (49.3)
Sex		
Female	118 (50.6)	277 (54.9)
Male	111 (47.6)	228 (45.1)
Unknown	4 (1.7)	--
Histologic Grade		
Well diff.*	4 (1.7)	161 (31.9)
Moderately diff.*	158 (67.8)	195 (38.6)
Poorly diff.*	45 (19.3)	44 (8.7)
Unknown	26 (11.2)	105 (20.8)
Stage		
I	45 (19.3)	107 (21.2)
II	59 (25.3)	124 (24.6)
III	94 (40.3)	194 (38.4)
IV	35 (15.0)	53 (10.5)
Unknown	--	27 (5.3)

Table 1. Summary of clinical and pathological characteristics of colorectal carcinoma cohorts. Asterisks indicate clinical / pathological features that differed significantly between cohorts ($p < 0.05$).

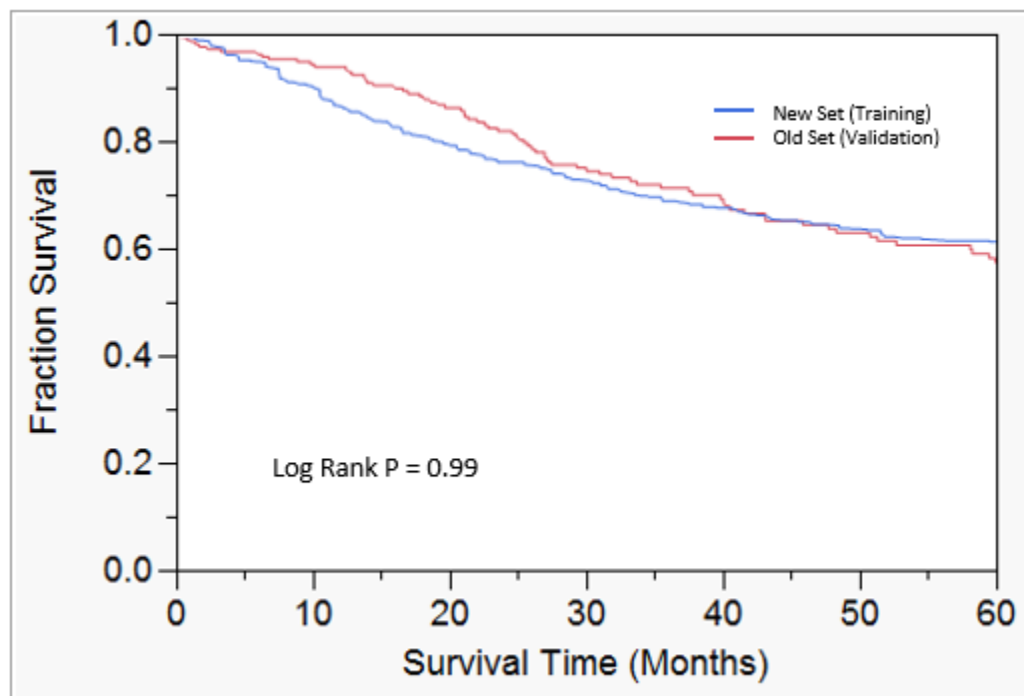


Fig. 1. Kaplan-Meier curve showing no difference in survival between CRC patients in the training (blue) and validation (red) sets.

in the dark, followed by preincubation with 0.3% bovine serum albumin (BSA) in 0.1 M Tris buffered saline (TBS, pH 8) for 30 min at room temperature. A primary antibody against CD68 (rabbit polyclonal ab125047; 1:750; Abcam, Cambridge, MA, USA) or TRAP (mouse monoclonal ab49507; 1:100; Abcam) diluted in 0.3% BSA/TBS was applied overnight at 4°C. After washing, slides were incubated with either an anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (EnVision; DaKo, Carpinteria, CA, USA) for CD68 or TRAP, respectively. The detection reaction was developed with DAB (DAB Enhancer; DaKo) for 5 min, then washed and counterstained with Tacha hematoxylin (Biocare Medical, Concord, CA, USA) for 1 min. Slides were then dehydrated in ethanol and mounted with xylene for 5 min, followed by coverslipping with Cytoseal 60 (Thermo Scientific, Waltham, MA, USA).

Immunofluorescent visualization of TRAP and CD68 co-staining was also performed on index arrays. Slides were deparaffinized and preincubated using the same procedures above. However, CD68 (rabbit polyclonal ab125047; 1:1000; Abcam) and TRAP (mouse monoclonal ab49507; 1:100; Abcam) antibodies were both diluted in 0.3% BSA/TBS during the primary incubation. Slides were then washed and incubated with a secondary antibody conjugated to a Cy3 fluorophore (Alexa 546 goat anti-rabbit; 1:100; Molecular Probes, Grand Island, NY, USA) diluted in an anti-mouse antibody conjugated to horseradish peroxidase (EnVision; DaKo). To allow visualization of TRAP, slides were then washed and incubated with Cy5 conjugated tyramide (1:50; PerkinElmer, Hopkington, MA, USA) for 10 min. Coverslipping was performed using Prolong Gold mixed with DAPI (Molecular Probes).

Multiplexing of TRAP with CD163 or iNOS was similarly performed on test arrays using the same deparaffinization and preincubation procedures. TRAP and CD163 (mouse monoclonal CD163-L-U; 1:50; Novocastra) or TRAP and iNOS (rabbit polyclonal ab15323; 1:200; Abcam) were diluted in 0.3% BSA/TBS for 30 min during the primary incubation. Slides were washed and first incubated with an anti-IgG1 secondary antibody conjugated to HRP (goat anti-mouse monoclonal 18-4015-82; 1:100; eBioscience), and then incubated with Cy3 conjugated tyramide (1:50; PerkinElmer, Hopkington, MA, USA) for 10 minutes to allow visualization of CD163. For multiplexing of TRAP and iNOS, slides were washed and incubated with an anti-rabbit secondary antibody (EnVision; DaKo). Slides were then quenched with 0.5 mM benzoic hydrazide solution with 0.5% H₂O₂, followed by incubation with an anti-IgG2b secondary antibody conjugated to HRP (goat anti-mouse monoclonal ab97250; 1:100; Abcam). To allow visualization of TRAP, slides were again washed and incubated with Cy5 conjugated tyramide.

Immunofluorescence of TMAs for AQUA analysis followed the same procedures as above. However, an antibody against pancytokeratin (rabbit polyclonal; 1:100; DaKo) was used instead of CD68 during the primary incubation to identify epithelium. Serial sections of an index array were also stained alongside each cohort to assess the inter-assay reproducibility.

Image Acquisition

Image acquisition for immunohistochemical arrays was performed using a ScanScope microscope (Aperio, Vista, CA, USA). Automated image capture of

immunofluorescence was performed using the HistoRX PM2000 device, as described previously.⁷⁵ Images of each histospot on the array were captured. Nuclear, CD68 or cytokeratin, and TRAP staining were visualized with DAPI, Cy3, and Cy5 channels, respectively.

Quantitative Immunofluorescence (QIF)

The AQUA method of QIF allows quantitative measurements of protein levels in subcellular compartments.^{75,76} Briefly, subcellular compartments are first defined using different fluorescent antibody tags to separate tumors from the surrounding stroma. AQUA then utilizes an algorithm that calculates the intensity of measured immunofluorescence within each subcellular compartment. In our case, a binary tumor mask is generated using cytokeratin staining as an indicator of tumor epithelial cells, and a cellular mask is generated from dilation of nuclei created from the DAPI staining. The tumor mask is then subtracted from the DAPI generated tissue and cellular mask, resulting in a new compartment that represents only the stromal tissue in each histospot (Figure 2). The signal intensity within this compartment is then divided by the area of this “stromal mask” in order to generate a TRAP AQUA score. These AQUA scores are then used for selection of cut-off points and subsequent analysis, as described below.

Statistical Analysis

For both cohorts, TRAP AQUA scores from two independent cores were averaged for final analysis. Optimal cut-off points for the training set were determined by X-tile, as described previously.⁷⁷ Kaplan-Meier curves, univariate, and multivariate Cox proportional hazards ratios were then generated using JMP 9 (SAS Inst, Glastonbury, CT,

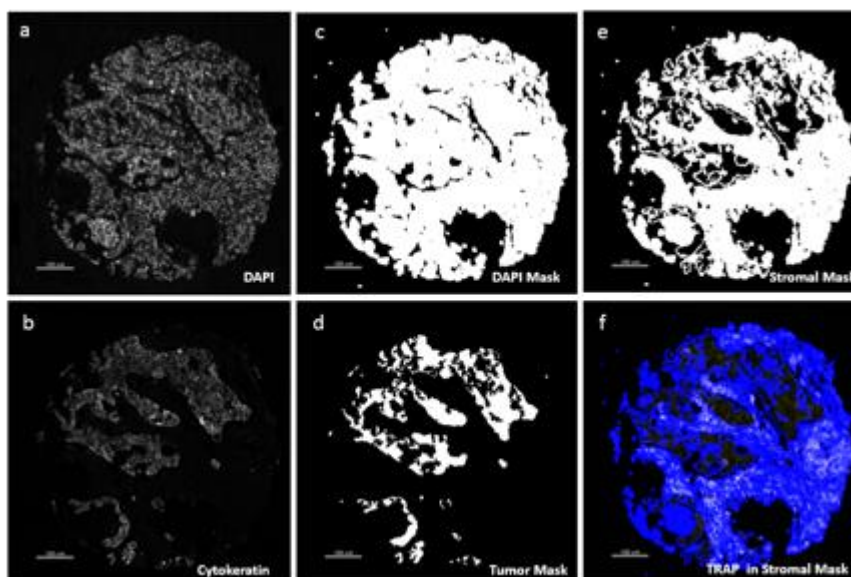


Fig. 2a-f Development of a stromal compartment in AQUA. (a) Nuclei stained by DAPI are dilated into a binary mask to create a (c) DAPI mask. As previously described, (b) cytokeratin staining is used to generate a binary mask of the epithelial compartment, called the (d) tumor mask. The tumor mask is subsequently subtracted from the DAPI mask to generate the (e) stromal mask. (f) Staining of the target TRAP (white) within the stromal mask (blue).

USA). Pearson's correlation coefficient (R^2) and linear regressions between near-serial sections of the index array were used to assess inter-array reproducibility, and to normalize the validation set to the training set. Following normalization, the cut-off point generated from the training set was applied to the validation set to generate a second Kaplan-Meier curve. A univariate and multivariate Cox proportional hazards analysis was performed to determine the prognostic value of TRAP while controlling for potential confounding factors. In addition, student t-tests were performed in the training and/or validation sets to determine differences in TRAP expression by stage and degree of tumor-infiltrating lymphocytes. All statistical analyses were conducted using JMP 9.

Results

Differential TRAP Expression Reveal Two Populations of Macrophages

We performed immunohistochemical staining on serial arrays of colorectal carcinomas to determine which cell types expressed TRAP. Positive TRAP expression was observed primarily in extra-epithelial tissue in cells with macrophage morphology. Figure 3a illustrates a representative example of a macrophage expressing TRAP. To determine whether TRAP is expressed in all macrophages, or whether it can distinguish differing sub-populations of macrophages, we assessed co-localization of TRAP in cells that were also expressing the common macrophage marker CD68. An example of routine identification of macrophages by immunostaining with CD68 is shown in Figure 3b. Figures 3c-e illustrate the results of immunofluorescent co-localization. As expected, TRAP staining (green) was observed primarily in stromal tissue, with several positive cells displaying macrophage morphology (Figure 3c). In addition, CD68 expression (red) displayed a similar staining pattern in the stromal tissue (Figure 3d). Merged images

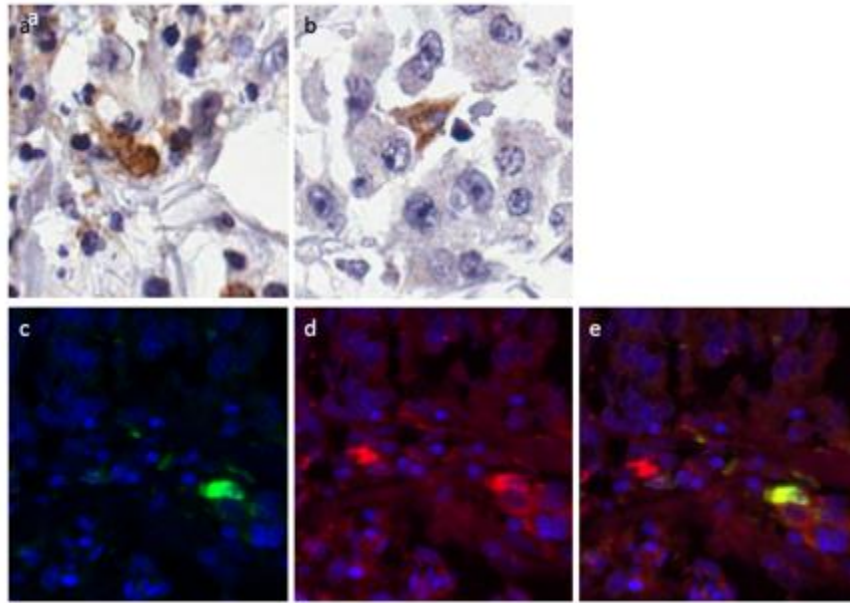


Fig. 3a-e Immunohistochemical and immunofluorescent photomicrographs of TRAP and CD68 expression on CRC tissue cores. Hematoxylin and positive diaminobenzidine (DAB) staining reveals macrophage expression of TRAP (a) and confirms CD68 as a marker for macrophages (b). (c) Immunofluorescent staining of TRAP (green) with DAPI nuclear staining (blue). (d) Immunofluorescent staining of the macrophage marker CD68 (red) with DAPI nuclear staining (blue). (e) Merged image of TRAP and CD68 staining indicating at least two populations of macrophages. Red indicates a CD68+ and TRAP- macrophage, while yellow indicates a CD68+ and TRAP+ macrophage.

however revealed that TRAP expression was not completely identical to CD68 expression. Although many cells demonstrated co-localized TRAP and CD68 expression, there were also several CD68⁺ cells that had no TRAP expression (Figure 3e). No CD68⁻ and TRAP⁺ cells were observed. This observation suggests that at least two populations of macrophages exist; those that express both CD68 and TRAP, and those that express CD68 but do not express TRAP.

To determine if TRAP expression is associated with a specific macrophage subtype, we assessed co-localization of TRAP with CD163, an M2 marker used commonly in the literature.⁴⁴ Figure 4c shows co-localization of TRAP with CD163, indicating M2 expression of TRAP. However, there is also evidence of TRAP⁺ and CD163⁻ cells (Figure 4f), indicating that TRAP is not exclusively expressed by M2 macrophages. Co-localization with TRAP and iNOS, an M1 marker used commonly in the literature, was also performed to determine if non-M2 macrophages were of the M1 phenotype.⁴⁴ Figure 5c shows co-staining of TRAP with iNOS, which appears to demonstrate co-localization of TRAP with iNOS. Unfortunately, iNOS antibody staining was complicated by non-specificity (see Figure 5b), making it difficult to draw conclusions on whether M1 macrophages express TRAP.

TRAP Expression and its Association with Favorable Outcome in Colorectal Cancer

Macrophages and inflammatory cells are considered to be stromal components that can affect the behavior of the adjacent tumor.^{27,32,33} Specifically M1 macrophages have been associated with tumor suppressive activity while M2 macrophages are tumor promoting.^{32,45} TRAP appears to be expressed in only a subset of macrophages, although

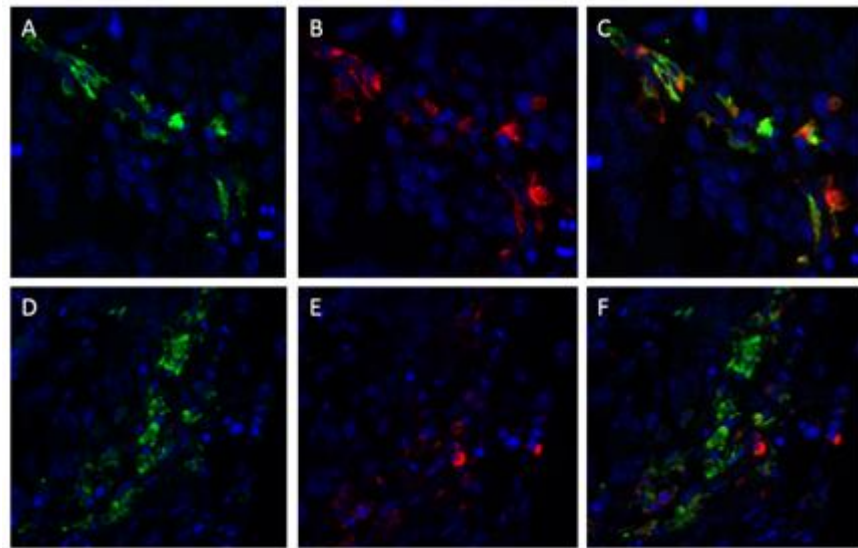


Fig. 4a-f. Immunofluorescent photomicrographs of TRAP and CD163 expression on CRC tissue cores (a, d) Immunofluorescent staining of TRAP (green) with DAPI nuclear staining (blue). (b, e) Immunofluorescent staining of the M2 macrophage marker CD163 (red) with DAPI nuclear staining (blue). (c, f) Merged image of TRAP and CD163 staining indicating both co-localization of TRAP and CD163 (c) as well as TRAP⁺ and CD163⁻ macrophages (f).

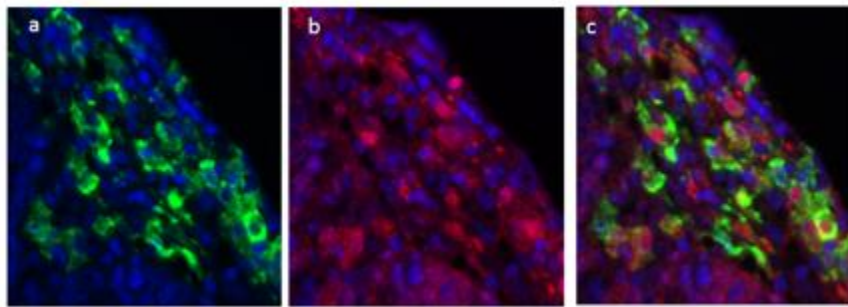


Fig. 5a-c. Immunofluorescent photomicrographs of TRAP and iNOS (1:200) expression on CRC tissue cores. iNOS staining was complicated by nonspecificity of the antibody. (a) Immunofluorescent staining of TRAP (green) with DAPI nuclear staining (blue). (b) Immunofluorescent staining of the M1 macrophage marker iNOS (red) with DAPI nuclear staining (blue). (c) Merged image of TRAP (red), iNOS (green), and DAPI nuclear staining (blue).

it has not been associated with a subtype. Since macrophages are localized to stroma, AQUA scores for TRAP were generated by measurement of signal intensity in the stromal compartment. The AQUA scores were averaged between two independent cores. Inter-array reproducibility was also measured by staining serial sections of an index array with colorectal carcinomas from a small sub-group of control patients. The Pearson's R^2 value for the index arrays between the training and validation set was 0.96 (Figure 6). The high Pearson's value indicates not only good experimental reproducibility, but also suggests relative homogeneity of TRAP expression in tumor tissue.

Measurement of expression of stromal TRAP in the newer colon cancer cohort showed a rightward skewed bell-shaped distribution (Figure 7a). Since there was no obvious cut-point in the distribution, we used a statistical method called X-tile, to define an optimal cut-point on the basis of overall survival.⁷⁷ Briefly, X-tile plots are generated by dividing a data set into three populations of high, middle, or low expression of a marker, with all possible divisions assessed. The plot is then visualized through a right-triangular grid, in which the X-axis represents all possible low populations (with population size increasing from left to right) and the Y-axis represents all possible high populations (with population size increasing from top to bottom). All data along the hypotenuse represents a single cut-off point that divides the data into high or low subsets, and can also be visualized in a strip below the grid. Data points on the grid are color-coded to represent the χ^2 value of each division. The brightness of the color represents the strength of the association, and points are colored green if the marker is positively

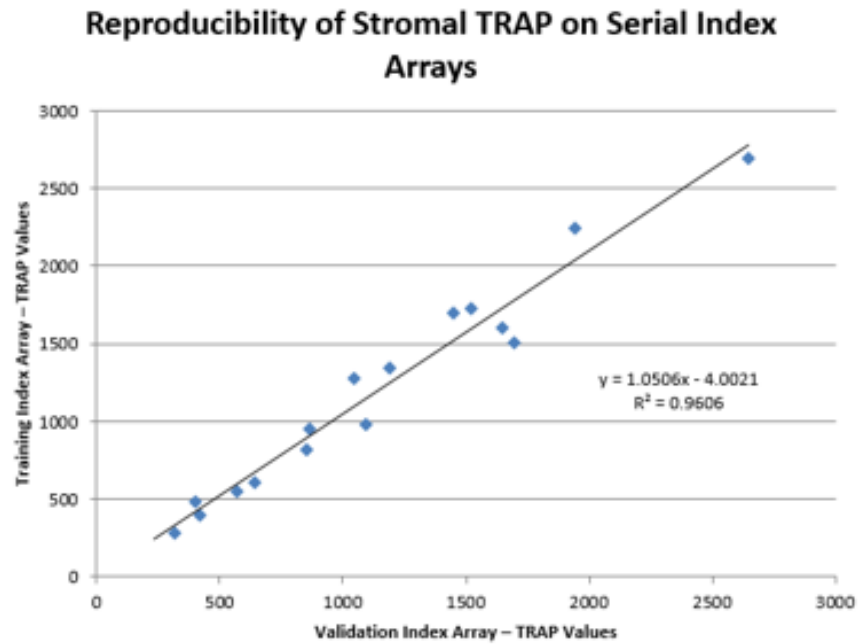


Fig. 6. Stromal TRAP expression on serial index arrays of CRC cores stained alongside the training and validation sets. The high Pearson's R^2 value (0.96) indicates reproducibility between staining and relative homogeneity of TRAP expression in tumor tissue.

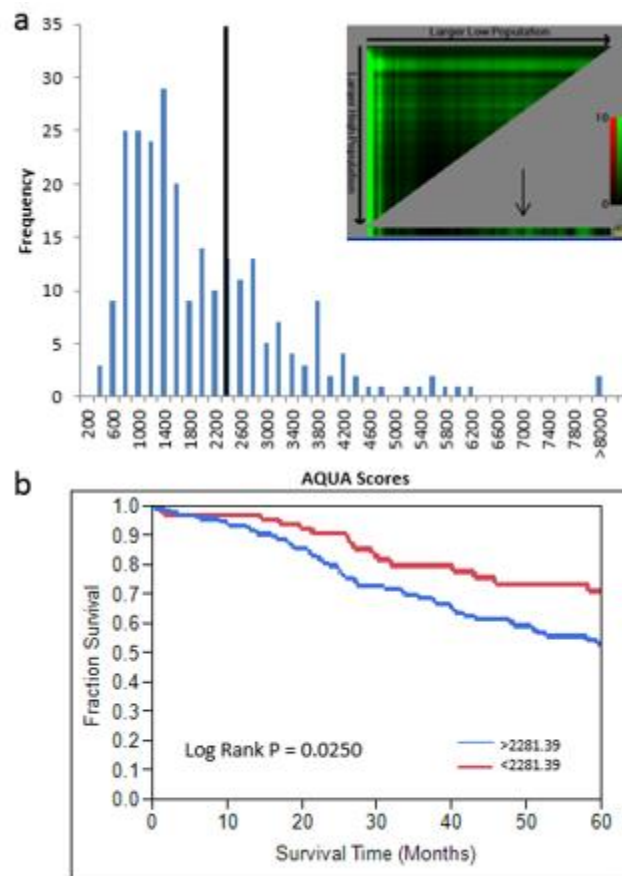


Fig. 7a-b (a) Histogram showing distribution of AQUA scores for stromal TRAP expression in the training set, divided into high and low populations. Inset shows the X-tile plot generated to determine the optimal cut-off (arrow; see text for details). (b) Kaplan-Meier curve of the training set showing differences in survival between patients with high and low TRAP expression using the generated cut-off point.

associated with survival, or red if negatively associated with survival. The optimal cut-point is shown as an inset in figure 7a and the resulting survival curve is shown in Figure 7b. The optimal cut-off point was determined to be an AQUA score of 2281 (uncorrected log-rank $p=0.025$), with 33.5% of patients in this cohort belonging to the “high” group (Figure 7a). A Kaplan-Meier curve was then generated to illustrate the differences in survival between the two groups (Figure 7b). Patients with high TRAP expression had a 22% increase in 5-year survival from patients with low TRAP expression (from 52.0% to 71.2%; uncorrected log-rank $p=0.025$).

Since the optimal cut-point was determined from all possible cut-points, correction is required for multiple testing. Rather than use a statistical approach, we selected a second, older, but larger cohort of colon cancer patients to serve as a validation set. The cut-off point was then applied to the validation set in order to determine if TRAP expression is significantly associated with favorable outcome. In the validation set, 15.8% of patients had TRAP expression higher than the normalized applied cut-off (Figure 8a). A Kaplan-Meier curve demonstrated a significant increase in survival for patients with high TRAP expression ($p = 0.0041$; Figure 8b). Overall, patients with high TRAP expression in the validation set had a 19% increase in 5-year survival (from 58.8% to 77.7%), confirming the initial findings in the training set. These results suggest that TRAP expression is correlated with favorable outcome in colorectal cancer.

Univariate and Multivariate Analyses Reveal TRAP Expression as Independent of Age, Gender, and Grade in Survival

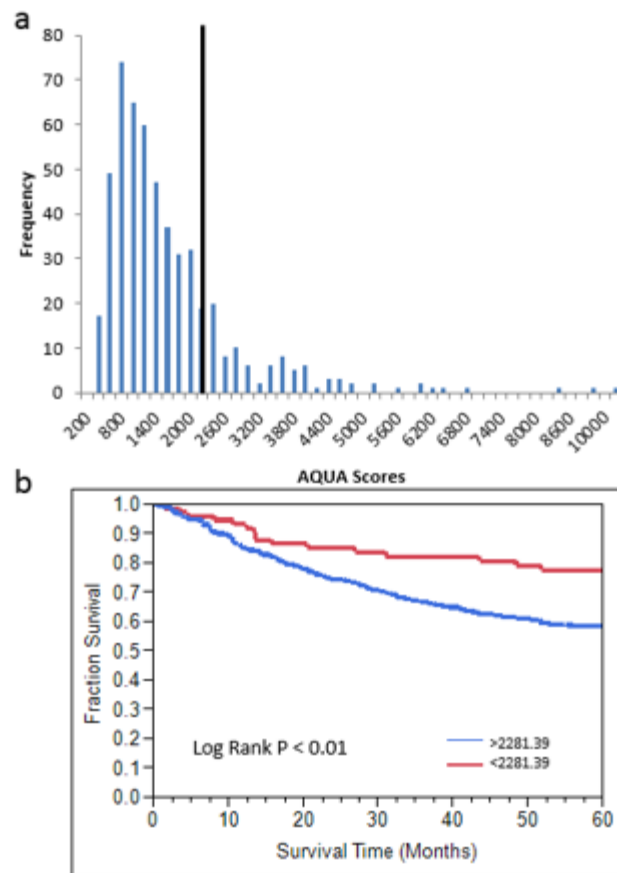


Fig. 8a-b (a) Histogram showing distribution of AQUA scores for stromal TRAP expression in the validation set, divided into high and low populations. The optimal cut-off point was previously generated from the training set. (b) Kaplan-Meier curve of the validation set showing differences in survival between patients with high and low TRAP expression using the generated cut-off point.

We performed univariate and multivariate Cox proportional hazards analyses to determine the prognostic value of TRAP while also controlling for potential confounding factors. In the new cohort, patients with high TRAP expression experienced a 47% risk reduction of death compared to patients with low TRAP expression (hazards ratio 0.53, $p=0.02$; Table 2a). In the older cohort, patients with high TRAP expression experienced a 52% risk reduction of colorectal cancer death compared to patients with low TRAP expression (hazards ratio 0.48, $p=0.0019$; Table 2a). When controlling for other prognostic factors such as age, gender, and grade, high TRAP expression was associated with a 46% reduction in death in the training set (hazards ratio 0.54, $p=0.04$) and a 51% reduction in colorectal cancer death in the validation set (hazards ratio 0.49, $p=0.05$; Table 2b). However, high TRAP expression was not significantly associated with decreased risk when stage was included in the analysis for both the training and validation sets ($p=0.06$ and $p=0.12$, respectively). We performed COX multivariate proportional hazards ratio analysis to determine the prognostic value of TRAP restricted to patients with Stages III and IV colorectal cancer only (Table 3). In YTMA221 (new set), high TRAP expression was associated with a 66% reduction in death, independent of stage ($p=0.0036$). In YTMA8 (old set), high TRAP expression was associated with a 50% reduction in death, independent of stage ($p=0.026$). In these analyses, age, gender, and grade were not significantly associated with risk of death (Table 2b).

Additional data on degree of tumor-infiltrating lymphocytes (TIL; absent, non-brisk, or brisk) was available for 199 patients in the training set. Given that multiple studies have shown that infiltration with CD4+ and CD8+ lymphocytes are associated with improved survival in colorectal cancer, statistical analyses were performed to

Table 2a. Univariate Analysis of TRAP Ratio		
	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
TRAP (High/low)	0.53 (0.29-0.91)	0.02
Old set (YTMA-8)		
TRAP (High/low)	0.48 (0.28-0.78)	0.0019

Table 2b. Multivariate Analysis of TRAP Ratio		
	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
Age (High/low)	0.77 (0.46-1.27)	0.30
Sex (F/M)	1.08 (0.65-1.79)	0.78
Grade (Poor/Well+Mod)*	1.80 (0.97-3.17)	0.06
TRAP (High/low)	0.54 (0.28-0.96)	0.04
Old Set (YTMA-8)		
Age (Above/low)	1.10 (0.69-1.76)	0.68
Sex (F/M)	0.83 (0.52-1.33)	0.43
Grade (Poor/Well)	1.65 (0.94-2.78)	0.08
TRAP (High/low)	0.49 (0.20-1.00)	0.05

*Due to the low number of patients with well differentiated carcinomas, patients with moderately and well differentiated carcinomas were combined together for grade analysis in the training set.

Table 2a-b (a) Univariate analysis of patients with high and low TRAP expression. (b) Multivariate analysis of patients with high and low TRAP expression, age, sex, and grade.

Table 3. Multivariate Analysis of TRAP Ratio in Stages III and IV Patients		
	Hazard Ratio (95% CI)	P Value
New Set (YTMA-221)		
TRAP (High/low)	0.34 (0.14-0.72)	0.0036
Stage (IV/III)	3.35 (1.84-6.03)	0.0001
Old Set (YTMA-8)		
TRAP (High/low)	0.50 (0.23-0.93)	0.0263
Stage (IV/III)	0.54 (0.31-0.87)	0.0105

Table 3. Multivariate analysis of high and low TRAP expression and stage in Stage III and IV patients.

determine if TRAP expression varied significantly with degree of TIL.⁸⁻¹⁰ In the training set, only one patient was recorded to have absent TIL, and so was removed from the analysis. Mean stromal TRAP expression in patients with brisk TIL (n=19) was 3804.27, while mean stromal TRAP expression in patients with non-brisk TIL (n=179) was significantly decreased at 1871.41 by student's t-test ($p<0.001$; Figure 9a). However, a Kaplan-Meier curve showed no difference in survival between patients with brisk or non-brisk TIL (Figure 9b).

Mean stromal TRAP expression was also determined according to patient stage in both the training and validation sets (Figure 10). Although TRAP expression decreased according to severity of stage, there were no significant differences found in TRAP expression between stages in both the training and validation sets using an ANOVA comparison ($p=0.10$ and $p=0.45$, respectively).

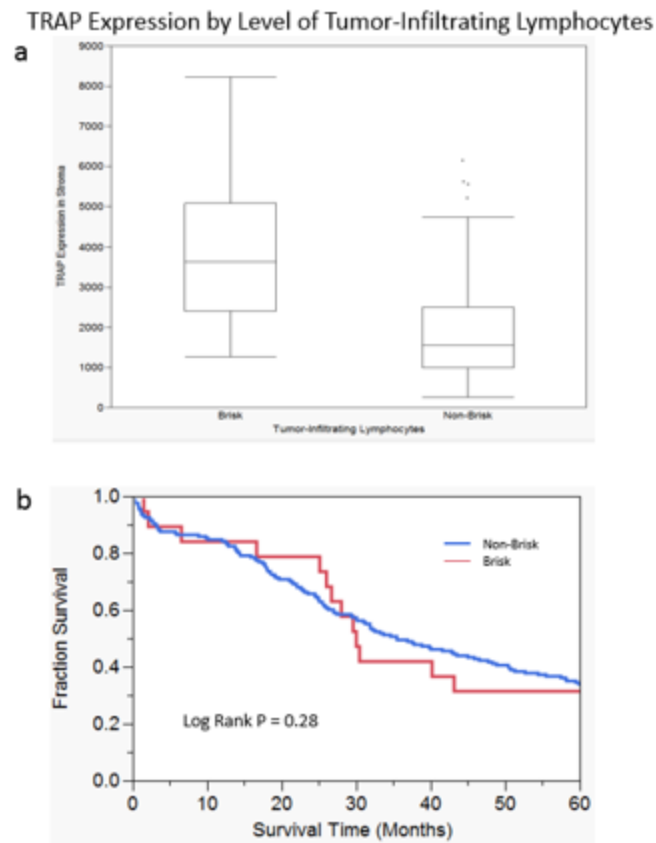
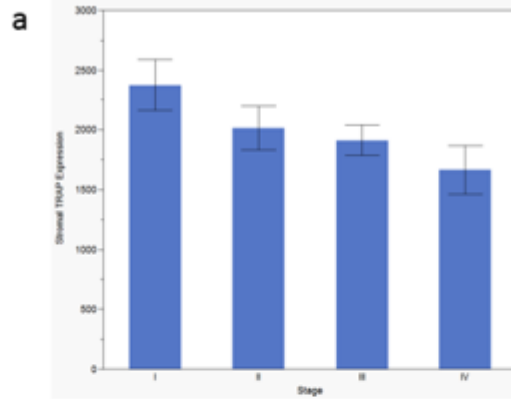


Fig. 9a-b Box plot showing distribution of TRAP scores for CRC cores with brisk (n=19) or non-brisk (n=179) tumor-infiltrating lymphocytes (TILs). CRC cores with brisk TILs showed mean stromal TRAP expression of 3804.27, while cores with non-brisk TILs showed mean stromal TRAP expression of 1871.41. Mean stromal TRAP expressions were significantly different ($p < 0.001$) by student's t-test. (b) Kaplan-Meier curve showing no difference in survival between CRC patients with non-brisk (blue) or brisk (red) TILs.

Stromal TRAP Expression by Stage (Training)



Stromal TRAP Expression by Stage (Validation)

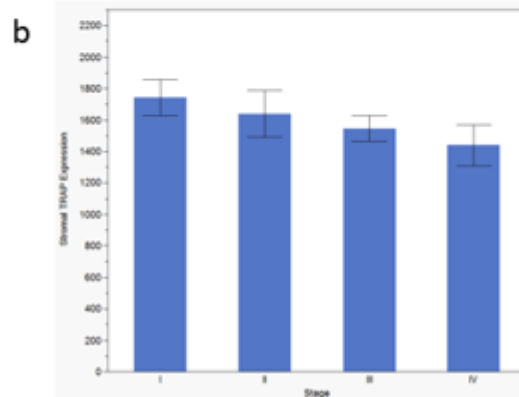


Fig. 10a-b (a) Mean stromal TRAP expression in training set. TRAP expression in Stage I, II, III, and IV CRC were 2378.43, 1916.42, 1472.81, and 1668.75, respectively. There were no significant differences in TRAP expression between stages ($p=0.10$). (b) Mean stromal TRAP expression in validation set. TRAP expression in Stage I, II, III, and IV CRC were 1742.12, 1640.03, 1547.25, and 1439.61, respectively. There were no significant differences in TRAP expression between stages ($p=0.45$).

Discussion

TRAP or ACP5 has not yet been well characterized with respect to its prognostic value, in spite of interesting mechanistic observations on the role of TRAP in tumor invasion⁶⁹ and in the host immune response to tumor invasion.^{32,65,68} We found TRAP expression in the stroma of tumor tissue, and showed morphological evidence that the expression was localized to a subset of macrophages using immunohistochemical stains. Furthermore, co-localization with CD68, a pan-macrophage biomarker, revealed that TRAP was able to distinguish separate populations of macrophages. Further co-localization of TRAP with CD163 revealed expression of TRAP in both M2 and non-M2 macrophages. Co-localization of TRAP with iNOS was suggestive of TRAP expression by M1 macrophages, although this observation was complicated by the general non-specificity of the iNOS antibody staining.

There is a great deal of evidence in the literature that CD68+ macrophages are associated with improved survival.⁷⁸ We were interested in whether certain subgroups of CD68+ cells (i.e. TRAP+ cells) are also associated with improved patient outcome. Survival analyses revealed that patients with high TRAP expression was significantly associated with improved 5-year survival rates and decreased risk of death. In addition, TRAP expression was increased in patients with brisk TIL and decreased stage, although the latter did not reach significance. These results indicate TRAP's potential use as a biomarker for favorable outcome in colon cancer, and add further complexity to the M1/M2 distinction in CRC prognosis.

To our knowledge, this is the first study to assess TRAP as a biomarker for macrophage activity in cancer. Previous studies have shown that TRAP is associated with poor outcome when expressed by tumor epithelial cells in melanoma, breast, and ovarian cancer,^{71,72} although the mechanism of action has not yet been elucidated. In addition, while previous research has confirmed TRAP's importance in the clearance of pathogens,⁶⁸ none have yet looked at TRAP's tumoricidal potential. It is believed that TRAP's importance in innate immunity derives from its ability to catalyze the production of ROS, which may contribute to its ability to kill cancer cells.^{65,66,68} Also, some evidence exists that substrates for TRAP are essential for microbial defense.^{69,70} However, TRAP expression by M2 macrophages may also indicate additional roles of TRAP that should be investigated further.

Given TRAP's mechanism of action in pathogen clearance, which is indicative of "classical" M1 macrophage activity, it is plausible that TRAP would be largely expressed by M1 macrophages. In addition, TRAP's association with improved prognosis in CRC is more suggestive of M1 expression, given previous studies that have shown more favorable outcomes and clinicopathologic features in patients with increased M1 macrophage infiltration. However, the results of our experiments showed that TRAP is expressed by both M2 and non-M2 macrophages. These findings are actually consistent with existing literature that suggest the M1/M2 macrophage distinction in cancer survival is not as clear-cut as originally proposed. For instance, although M1 macrophages are associated with increased survival, researchers have found that in these same cohorts that M2 macrophage infiltration similarly increases survival in CRC.^{22,56} This is possibly due to the fact that increased M2 macrophage infiltration was also associated with a

concomitant increase in M1 macrophage infiltration, although Edin et al. found no significant differences in survival when comparing differing ratios of iNOS/C163.⁵⁶ However, it is possible that given M1 infiltration is similarly increased in patients with increased M2 infiltration, the anti-tumorigenic effect of M1 macrophages may dominate over the tumor promoting effect of M2 macrophages.⁵⁶

However, the finding of M2 expression of TRAP suggests increased complexity in the role of M2 macrophages on tumorigenesis, which may also mediate the above findings in CRC outcome. For one, the designation of “M2 macrophage” refers to a diverse subpopulation of cells that may in fact have important functional and phenotypical differences. Indeed, further research into M2 macrophages have revealed at least three additional subgroupings based on activating stimuli and cytokine profiles, denoted M2a, M2b, and M2c.⁷⁹ M2a macrophages are primarily involved in tissue repair, while M2b macrophages are more heavily involved in antibody responses to infection; M2c macrophages, on the other hand, are anti-inflammatory and implicated in down-regulating the M1 response.^{32,79} It is possible that given the diversity of the M2 macrophages, each specific subtype has its own unique role in tumorigenesis as well. However, most of the existing literature on M2 subcategorization has focused primarily on characterization, with little work done in the context of cancer survival and outcome.^{57,79} TRAP expression may denote a particular type of M2 macrophage that differs from the activities usually associated with TAMs. Additional research on both the mechanisms of action of TRAP and specific M2 subpopulations in tumorigenesis would thus be merited.

Concomitant M2 and non-M2 expression of TRAP also provides evidence for the blurring of the M1/M2 distinction. Although originally proposed as a dichotomy, there has been increasing evidence that there is considerable plasticity of monocyte differentiation, and M1 versus M2 activation may actually represent the extremes of a spectrum.³² For instance, Edin et al. observed a small population of macrophages that were positive for both CD163 and iNOS expression, although they noted that this co-expression represented a minority.⁵⁶ Using flow cytometry and intracellular staining of M1 and M2 associated proteins, Patil et al. demonstrated the co-existence of M1 and M2 programs within the same macrophage cells responding to *Toxoplasma Gondii* infection; furthermore the proportion of M1, M2 and double positive macrophages would fluctuate depending on the time point after initial infection.⁸⁰ Macrophages recruited to an inflammatory site therefore may have the capacity to locally convert to either end of the M1/M2 spectrum, depending on the tissue microenvironment.⁸¹ This would have important implications in cancer immunotherapy.⁵⁸ The expression of TRAP by both M2 and non-M2 macrophages supports macrophage heterogeneity as a continuum. Thus, it may be more appropriate to characterize an individual macrophage as a mixture of both M1 and M2, depending on where in the continuum it falls. It would be interesting to perform more quantitative studies of TRAP expression by M1 versus M2 macrophages, as our study was a primarily qualitative one.

TRAP was also found to be significantly increased in CRC patients with brisk TILs. This is consistent with studies that have shown improved survival in patients with brisk versus non-brisk TILs, although we failed to show a survival benefit in our training cohort.⁸ This may have been due to the relatively small number of patients with brisk

TILs (n=19). Thus, increased TRAP expression is likely one component of the successful immune response against tumor cells. Our data supports the possibility that TRAP represents an alternative biomarker indicative of an appropriate host response to tumor detection, although it is not based exclusively on M1 or M2 activation. In addition, because TRAP activity may have a direct effect on tumoricidal activity, further exploration of TRAP's mechanisms of action may open up avenues for immune-mediated therapeutics.

Given TRAP's possible role in pathogenesis and progression of the tumor, it would have been expected to see decreases in TRAP with increased stage. Although there is certainly a trend in that direction, there are no significant differences found by ANOVA comparison of TRAP between stages (although an individual student t-test in the training set did note a significant difference in TRAP values between Stage I and Stage IV tumors, with $p=0.01$). It could be that a larger sample size would be needed to see the effects of TRAP with staging. In addition, the generated cut-off values of TRAP expression are higher than the mean TRAP scores for each stage in the training and validation sets. Thus, it is possible that while high TRAP expression indicates an effective host response to tumorigenesis (and predicting good prognosis), at lower values TRAP expression may fail to differentiate survival among different tumor stages.

It would therefore be interesting to see if TRAP is predictive of cancer progression in pre-cancerous lesions. There have been limited studies looking at macrophages and tumor progression in adenomas with dysplasia, although existing literature has shown that macrophage physiology certainly contributes to tumorigenesis in premalignant lesions. For instance, the increased risk of CRC in patients with

inflammatory bowel disease highlights the key role of inflammation and tumor progression, which is partly mediated by macrophages in the tumor microenvironment.⁸² In addition, the link between aspirin use and the prevention of CRC can be explained through inhibition of COX-2 expression in interstitial cells such as macrophages. In one small study, Bamba et al. found co-localization of COX-2 expression and CD68 staining in human CRC adenomas, but not in adjacent normal colonic mucosa.⁸³ However, how these inflammatory macrophages fit into the categorization of M1 versus M2 phenotypes has not been clearly studied.

Future research should focus on understanding TRAP's mechanisms of action in the context of its tumoricidal effects. While there have been a handful of studies in the literature about TRAP's anti-pathogenic effects, there have been no known studies of how it may exert its effects against tumor cells.^{65,68-70} More quantitative studies to better demarcate the proportion of M1 and M2 macrophages expressing TRAP would also further elucidate both TRAP's function and the M1/M2 distinction. In addition, it would be interesting to extend TRAP's association with outcome to other cancers, especially in cancers that are historically associated with TAMs that have negative effects on survival. It is possible that TRAP may be significantly decreased in cancers in which the host immune response is negatively altered by the tumor environment. The role of TRAP in preventing tumor spread and metastases should also be pursued. Unlike in breast cancer, increased macrophage infiltration is associated with decreased incidence of liver⁸⁴ and peritoneal⁸⁵ metastases. The mechanism is unclear, but authors have speculated that this may again be due to differences in M1/M2 phenotypes.⁸⁴ Evaluating TRAP expression in this context may also shed further light on this phenomenon.

There are a number of limitations in this study. We focused on TRAP's effect on survival outcome in colorectal cancer and used primarily morphological assessment of expression to investigate which subtypes of macrophage populations express TRAP. Given TRAP's expression in M2 macrophages, it is unclear what precise role TRAP plays in tumorigenesis. In our study, we did not observe any CD68- TRAP⁺ cells, but the presence of non-macrophage cells with positive TRAP expression cannot be discounted, as dendritic cells have been known to express TRAP.⁶⁹ Thus, it is possible that TRAP's association with improved survival may also be due to expression by other immune cells.

A second limitation of the work is that it was performed entirely on tissue microarray cohorts. While this is now a common approach, future studies will be required to determine if conventional slide analysis of TRAP is consistent with the observations we have made on the tissue microarrays. In addition, further validation of TRAP's prognostic role in larger, multi-institutional cohorts should be considered.

In addition, there may be sources of intrinsic bias present in our study. Although the cohorts were assembled independently of one another, the frequencies of each stage were very similar to one another, and both cohorts were predominantly Caucasian although in the general population African American patients have a higher incidence of CRC. Although the frequencies of each stage are roughly equivalent to those found in the general population, the Caucasian predominance does raise the issue of generalizability outside these two cohorts. The samples may also be skewed toward resectable tumors and tumors that are large enough for TMA assembly, such that very small tumors or widely metastatic tumors may not be included in the analysis.

Because the optimal cut-off value was statistically generated from all possible cut-offs, there is a risk that our proposed cut-off was a false positive. However, application of this cut-off to an independent validation cohort showed a robust correlation with survival. Clinical validation in further cohorts would be necessary to reliably show TRAP's association with improved outcome. This study investigating TRAP as a prognostic biomarker candidate is exploratory, corresponding to Phase 1 of the NCI's biomarker discovery pathway.⁸⁶ Of note, the biomarker discovery pathway proposed by Pepe et al. deals specifically with biomarkers developed for the detection of preclinical disease, rather than prediction or prognostication.⁸⁶ Several parallels can be drawn regarding the next steps for formalizing TRAP as a prognostic biomarker. In Phase 2 of the biomarker discovery pathway, a clinical assay that reliably distinguishes patients with different outcomes should be developed.⁸⁶ Staining for TRAP is already used in the clinical setting for diagnosis of hairy cell leukemia. However quantification of TRAP has yet to be translated into the clinical setting. Further studies should focus on how the proposed TRAP cut-off corresponds to visual staining of tumor samples, with possible development of a more accessible clinical score. Validation of this score in larger populations would then be necessary to assess TRAP's viability as a useful prognostic biomarker.

In conclusion, this study demonstrates a novel application of TRAP in the prognosis of colorectal cancer, and provides further evidence for subclasses of macrophages with differential roles in the tumor microenvironment.

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